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(54) Title: METHOD FOR THE IN VITRO PRODUCTION OF PROTEIN FROM A DNA SEQUENCE WITHOUT CLONING

(57) Abstract

In vitro production of protein from DNA sequences may be obtained utilizing a new and unique experimental technique called expression-PCR (E-PCR) which does not require cloning of the DNA segment of interest into any plasmid or phage vector. A universal promoter was developed containing an untranslated leader sequence from alfalfa mosaic virus directly downstream from the T7 bacteriophage promoter. When this universal promoter is spliced to a DNA segment in the appropriate way it produces a suitable template for in vitro transcription and translation. The DNA to be expressed is first amplified by the polymerase chain reaction (PCR) using a 5'-primer that incorporates an area homologous to the 3'-end of universal promoter. The universal promoter and this DNA fragment are mixed and re-amplified in a reaction analogous to splicing by overlap extension, generating a modified universal promoter linked to a DNA sequence that can now be transcribed and translated efficiently in vitro without further processing.

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METHOD FOR THE IN VITRO PRODUCTION OF PROTEIN FROM A DNA SEQUENCE WITHOUT CLONING.

GOVERNMENT INTEREST

FIELD OF THE INVENTION

This invention pertains generally to the field of molecular biology and particularly to the production of RNA and protein in vitro.

BACKGROUND OF THE INVENTION

The in vitro transcription and translation of DNA segments, or genes, are powerful tools to examine the structure-function relationship of proteins. The in vitro transcription of DNA into mRNA and its translation into protein is now done by cloning the DNA of interest into a plasmid which contains a promoter site for binding of a RNA polymerase protein. The recombinant DNA procedures for this cloning, while fairly straightforward, are time consuming, expensive and tedious and as a consequence extensive recombinant manipulation of cloned DNA is kept, in reality, to a minimum. The cloning procedure requires isolation of the DNA fragment, genetic engineering of proper ends onto the molecule, ligation into a enzyme digested prepared plasmid, transformation of that ligated DNA into bacteria, selection of transformants and then

purification of the ligated plasmid. This plasmid must then be digested with a suitable enzyme at a point in or after the gene in order to end transcription. Plasmid is then purified and in vitro transcribed into mRNA. This mRNA is translated into protein. The disadvantage of the above described in vitro manipulations is that the entire procedure can take as long as two or more months for each single construct that is made.

One approach, for example, to express a gene fragment would involve the following steps. First the DNA fragment would have to be purified. This can be done by restriction digestion or by the polymerase chain reaction (PCR). Second, the ends of the DNA fragment would have to be modified to be compatible with the end sequences in the cloning vector. If a suitable start signal or "ATG" codon did not exist one would have to add this. Suitable vectors for transcription of DNA fragments are commercially available (Jendrisak, et al., patent # 4,766,072). These usually contain the T-7 or SP-6 polymerase sites but not a ribosome binding site or enhancers of translational activity as has been reported (Jobling, S.A. et al., (1987) Nature:325, 622-625). Therefore a ribosome binding site has to be added to the fragment and if desired, a sequence which can act as an enhancer of translational activity can be added. After all these manipulations are done the modified DNA fragment is ligated into the prepared vector plasmid by the use of the enzyme T-4 ligase and transformed into bacteria by electroporation or absorption. Bacteria which have taken up plasmids are identified by plating on nutrient agar plates containing an antibiotic to which resistance is confirmed by an antibiotic resistance gene on the same plasmid. Single colonies are isolated and analyzed for plasmids with inserts of the desired DNA fragment. Once a clone is selected it is grown, usually in liter quantities, and the

plasmid purified from the bacteria by ultracentrifugation and CsCl banding. The purified DNA, now in a closed circular configuration, is digested with an appropriate restriction enzyme to linearize it before transcription.

Transcription, or the synthesis of RNA off a DNA template, is accomplished with the use of a RNA polymerase such as T7 RNA polymerase. The other reagents needed are the linearized DNA template, salts, buffer and ribonucleotides. Commercial kits are available to perform this synthesis. Once the RNA is made, if it contains an open reading frame of codons it may be translated into a protein. This is done in vitro with the aid of wheat germ extract, rabbit reticulocyte lysate or bacterial lysate extract, all commercial available or easily made by a competent technician. Such protein can be made with the incorporation of radiolabelled amino acids to aid in the analysis of the product.

The discovery of PCR (Mullis, et al., patent # 4,683,202) made it possible to amplify portions of DNA without the need for cloning (Saika, R.K., et al. (1988) Science 239:487-491). The key reagent in the PCR reaction is a thermostable DNA polymerase (Taq polymerase) isolated from Thermus aquaticus. With the selection of the appropriate oligonucleotide primers which flank the desired segment of DNA to be amplified, appropriate buffer, and deoxynucleotides, a single DNA segment can be amplified a million fold. The use of this method and the uniqueness of the universal promoter used in the manner outlined by this invention allow the implementation of the method described here as E-PCR to preform the above manipulations in one to two days.

SUMMARY OF THE INVENTION

In accordance with the present invention, it is possible to make in vitro a DNA construct that contains a site of RNA polymerase binding, an enhancer of translational activity, a ribosome binding site and a "start codon" or ATG sequence in front of any desired DNA fragment in order to obtain RNA copies which can be translated in vitro.

It is, therefore, also, an object of the present invention to make these gene fragments quickly and easily, by one reasonable skilled in the art of recombinant DNA technology, without the need to clone the DNA fragment into a plasmid vector.

It is a further object of the present invention to describe a double stranded DNA fragment, which we call a "universal promoter" which is spliced to the DNA fragment of interest by the process of splicing by overlap extension (SOE), which allows in vitro transcription of a mRNA molecule which has the required elements for efficient translation into protein.

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is the double stranded sequence of the first construct of universal promoter (UP-1) showing the site of binding of the T7 RNA polymerase, the untranslated leader sequence (UTL) from alfalfa mosaic virus (AMV) and the triplet codons for the amino acids MET and ALA.

FIG. 2 is the double strand sequence of the what is generated when UP-1 is spliced to DNA fragment of interest by SOE using primer H3T7 as the forward primer.

FIG. 3 is the double strand sequence of the second construct of a universal promoter (UP-3) which differs from UP-1 by the modification of the 3' end.

FIG. 4 is the double strand sequence of the what is generated when UP-3 is spliced to DNA fragment of interest by SOE using primer H3T7 as the forward primer.

FIG. 5 is the sequences of the oligonucleotide primers used in the construction and testing of the universal promoters

FIG. 6 is a diagrammatic outline of the steps used in the expression PCR reactions. The double stranded DNA and oligomers are represented by lines and arrows indicating the 5' to 3' orientation. Oligomers are denoted by bold upper-case letters and amplified products by the steps 1-3. The univeral promoter is first made from primers pUP1 and pUP3 (step 1). The gene to be expressed is amplified from genomic or plasmid DNA using primers A and B. Because primer A has nucleotides at its 5' end complimentary to the 3' end of the universal promoter the amplified product (step 3) has a region homologous to the 3' end of the universal promoter at its 5' end. The products of step 1 and step 2 are mixed with primer H3T7 primer B (boxed reaction) and amplified in a two step PCR analogous to splicing by overlap extension. Initially no primers are added and each DNA strand acts as primer and a template for the other to produce the recombinant molecule composed of the T7 promoter and the untranslated leader sequence spliced in frame to the gene of interest. This molecule used as a template for in vitro transcription and translation without further processing (step 4).

FIG. 7 is an immunoprecipitation of E-PCR product of a "repeatless" gene construct of the P. falciparium CS gene. mRNA was produced by the E-PCR method

and translated in a rabbit reticulocyte lysate cell-free system in the presence of [^{35}S] and analyzed by 0.1% SDS - 15% PAGE and autoradiography (lane a). Translated repeatless CS protein was immunoprecipitated only by antisera against the repeatless portion of the protein (lane c) and not by antibodies to the repeat region (lane b) or by antibodies specific for the CS protein of P. berghei (lane d) or by protein A alone (lane e).

Fig. 8. is the analysis of two different E-PCR constructs of the EBA-175 gene. Immunoprecipitation and specific binding to RBC's are shown. The EBA-175 mRNA's produced by the E-PCR method were translated in a wheat germ cell-free system in the presence of [^{3}H]leucine and analyzed by 0.1% SDS - 15% PAGE and autoradiography (lanes 1 and 3). Translated protein was immunoprecipitated by antipeptide 4 sera (lane 2) which recognized a peptide within this construct. Full length protein product made in lane 3 bound to RBC's susceptible to invasion by P. falciparum merozoites (lane 4).

DETAILED DESCRIPTION OF INVENTION

The above objects and advantages of the present invention are achieved by the use of the "universal promoter" in the procedure described by us as expression polymerase chain reaction (E-PCR). This invention allows selective in vitro transcription of DNA in accordance with the invention without the need to clone the piece of DNA into a plasmid vector. The active polymerase binding site (FIG 1,

positions -1 to -17) described in this invention is the one for viral T7 RNA polymerase, however, any promoter site may be used that corresponds to the RNA polymerase that will be employed for the transcription of the DNA. Promoter sites for suitable polymerases that could be used are those for the SP6 polymerase, the T3 or N4 phage polymerase or the ghl promoter. Many other polymerases and the promoters they recognize can be used in accordance with the invention.

The untranslated leader (UTL) sequence between the T7 promoter and the initial ATG codon (FIG. 1, positions +1 to +38) is derived from the coat protein mRNA of the alfalfa mosaic virus (AMV). In vitro translation of mRNA is often dependent on the presence of, and characteristics of, an UTL sequence 5' to the initiation codon. It has previously been shown that replacement of a gene's native UTL with the UTL sequence of AMV can increase translational efficiency as much as 35-fold (Joblin, Nature, id.). This enhanced expression may be explained in part by a decreased requirement for translational initiation factors by mRNA containing the AMV UTL sequence. Additionally, the efficiency of translation may be poor if the AUG (in the mRNA) initiation codon lies too close or too far from the 5'-end of the mRNA (Struhl, K. (1989), Current Protocols in Mol. Biol., 10.17.1-10.17.5) if the initiation codon resides in a poor sequence context (Kozak M., (1986) Cell 44:283-292); is inaccessible due to secondary structure of the mRNA (Pelletier, J. et al., (1985) Cell 40:515-526); or if there are increased requirements of translation initiation factors (Browning, K.S. et al, (1988) J. Biol. Chem. 263:9630-9634). Other UTL sequences could be substituted for this AMV UTL.

The number of nucleotides required upstream of position -17, the first specific nucleotide required for T7 RNA polymerase binding, is not known. The least number of nucleotides shown to be required is 5 (Milligan, J.F., et al, (1987) Nucleic Acids Res. 21:8783-8798; Ikeda, R.A., et al. (1986) PNAS 83:3614-3618). Tests to determine a number fewer than this have not been reported. Milligan (1987, Nucleic Acids Res. id.) reports that unlike E. coli RNA polymerase the presence of long, non-specific 5' flanking DNA does not lead to significant rate enhancement of the polymerase activity either. Footprint analysis (Ikeda, PNAS, id.) indicates that the sequence of the upstream fragments is not critical except that at least 5 nucleotides are probably needed to stabilize the protein-DNA interaction of the polymerase with the promoter site. The original construction of the UP-1 (FIG.1) contained only 3 nucleotides upstream from the -17 nucleotide. This construct did not give efficient transcription of the downstream DNA into RNA. In order to lengthen the upstream sequence we designed primer H3T7 (FIG. 5) which adds nine nucleotides upstream of the -17 position. This primer was used in the third step of the E-PCR reaction (EXAMPLE 3, below) and resulted in UP-2 which gave increased transcriptional activity. The nucleotides selected for this extension contain the site of the Hind III restriction endonuclease only for future considerations of cloning the UP, and are not meant to be specifically needed. Further minor modifications in the universal promoter sequence were made at the 3' end to facilitate primer design for the SOE reaction (see EXAMPLE 1).

The term "universal promoter" (UP) as used herein is intended to indicate a double stranded DNA fragment which has the characteristics of nine nonspecific base

pairs of nucleotides 5' to the known sequence for initial binding of a mRNA polymerase, like but not limited to T7 RNA polymerase, followed by 38 nucleotides describe as an "untranslated leader (UTL) sequence" derived from the coat protein mRNA of the alfalfa mosaic virus (AMV) and ending in a 7 (UP1) or 9 (UP3) nucleotides base pairs beginning with "ATG" initiation codon.

The following examples are provided as illustrative of the methods used for the generation of DNA fragments in accordance with the invention, the construction of the universal promoter, the splicing of the universal promoter to a desired DNA fragment, the methods for in vitro production of RNA coded by the DNA and its translation in vitro into protein.

EXAMPLE 1

Construction of the Universal promoter

A universal promoter-1 having the sequence shown in FIG. 1 was constructed by primer-dimer formation in a polymerase chain reaction (Saika, R.K., et al. (1988) Science 239:487-491; Browning, K.S., (1989) Amplifications 3:14-15,) from the primers pUP1 and pUP2 shown in FIG. 5. The two primers pUP1 and pUP2 were synthesized on an Applied Biosystems 380B DNA synthesizer, deprotected by ammonium hydroxide treatment and desalted by passage over a Pharmacia PD-10 column containing Sephadex G-25 as described (Jayaraman, K., (1987) Biotechiques 5(7):627). They were added together in a PCR reaction and because their last five 3' nucleotides were complementary, amplified each other in a primer-dimer formation reaction. The double stranded universal promoter-1 was applied to an agarose gel to electrophorese away unextended single stranded primers. The double stranded universal promoter-1 was visualized by ethidium bromide staining and the area of the gel containing the universal promoter-1 band was excised and stored in a 1.5 ml polypropylene microcentrifuge tube. The sequence of pUP1 was designed to include the T7 RNA polymerase binding site and part the of UTL sequence of AMV. The sequence of pUP2 was designed to include the remaining complementary downstream sequence of the AMV UTL with a five base overlap complementary to the UTL sequence of pUP1, and a start translation 'ATG' codon. An additional feature was the design of a Nco 1 restriction endonuclease site around the 'ATG' codon to facilitate future cloning if desired (FIG 1.).

Further modifications were made to the invention to improve it. In the design of the splicing primer forgetting to add two bases between the universal promoter-1 specific sequence and the gene specific sequence often lead to primers that did not work correctly in the invention. Therefore the universal promoter-1 was redesigned with these two nucleotides added to the 3' end by making a new reverse primer pUP3 (FIG 5) for the primer-dimer construction of a universal promoter-3. This construct had the added improvement of using additional cytosines and guanines to increase the T_m of the overlap in the SOE reaction by changing the codon usage for the LEU amino acid. Additionally, in order to increase specificity in the splicing by overlap extension step of the procedure the universal promoter-3 was redesigned to include nine base pairs upstream from the -17 site of the T7 promoter binding site (Fig 4) and called universal promoter-4.

Specifically, 100 pmols of pUP1 and pUP2 were added in a final 100ul reaction containing 200 uM each dNTP (dATP, dGTP, dCTP, dTTP), 10ul 10X reaction buffer (10X reaction buffer consists of 100mM Tris-HCl, pH8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin) and 2.5 units Taq DNA polymerase. The reaction was amplified by 20 cycles each of 2 min at 94° C, 2 min at 50° C, 2min at 72° C. The last cycle was followed by an incubation at 72° C for 7 min. The reaction was extracted one time with 100 ul of chloroform, precipitated by adding 50 ul of 7.5M ammonium acetate and 2 vols of 100% ethanol and placing at -20° C for 30 min. The sample was centrifuged, the precipitate washed with cold 80% ethanol and resuspended in 20 ul of TE (10mM Tris, pH 8.0, 1mM EDTA), and loaded onto an agarose gel consisting of 2% NuSieve

agarose, 1% Seakem agarose, 0.5ug/ml ethidium bromide, 1X TAE buffer (10X Buffer is 0.4M Tris Base, 0.2M sodium acetate, 10mM EDTA, pH7.2). The gel was electrophoresed for 30min in 1X TAE buffer and the band excised and stored in a 1.5ml polypropylene microcentrifuge tube at -20° C.

EXAMPLE 2

PCR of the DNA of interest

Synthesis of the gene segment of interest was done by the standard PCR technique. Oligonucleotide primers for the PCR reaction were synthesized and purified as described in EXAMPLE 1. The key to designing the primers required for this invention was to include on the 5' end of the splicing primer A (FIG. 5) the same sequence of 7 nucleotides as the last 7 nucleotides on the 3' end of the universal promoter-1 with the addition of two bases so the gene segment of interest was in the correct reading frame with the start of translation signal (ATG) of the universal promoter-1. The bases chosen were selected to give the amino acid LEU upon translation. If universal promoter-3 or universal promoter-4 were to be used the 5' end of the splicing primer A needed to be the same as the last 9 nucleotides on the 3' end of the universal promoter. The remaining 18 nucleotides of splicing primer A were specific to the DNA segment of interest, in this example a gene segment on the coding strand of EBA-175 (Sim, et al 1990). The sequence of the reverse primer was specific to a downstream segment of the same gene but on the complementary strand. The

double stranded piece of specific DNA that was amplified and because of the design of splicing primer A could be spliced by overlap extension PCR (SOE-PCR) (Horton, R.M., et al, (1989) Gene 77:61-68) to the universal promoter.

Specifically, the splicing primers A and the reverse primer B (FIG. 5) were synthesized on an Applied Biosystems DNA synthesizer (model 380B) and deblocked with ammonium hydroxide treatment and desalted over a PD10 column. For the PCR reaction 50 pmol of each primer were added in a final 100ul reaction with 10 ng template DNA (the EBA-175 gene cloned into a plasmid), 200 uM each dNTP, 10ul 10X reaction Buffer and 2.5 units Taq DNA polymerase. The reaction was amplified in an automated thermal cycler (Perkin Elmer Cetus) using 25 cycles (each consisting of 2 min at 94° C, 2 min at 50° C, 2 min at 72° C) followed by a 7 minute incubation at 72° C. The PCR products were separated on a 2% LMP NuSieve agarose gel and the DNA bands were excised and stored at 4° C until spliced to the universal promoter by a SOE-PCR reaction.

EXAMPLE 3.

Splicing by overlap extension of the UP to the gene fragment of interest

In order to splice the universal promoter to the DNA fragment of interest the agarose containing the universal promoter made in EXAMPLE 1 and agarose containing the gene of interest, made in EXAMPLE 2, were melted at 60° C, and 2 ul of melted agarose containing 25ng of each DNA were added together, without primers, in a single PCR reaction of a final 90 ul volume containing 9 ul 10X reaction buffer and 200 uM each dNTP, 1.3 units Taq polymerase for 15 cycles (each consisting of 2 min

at 94° C, 2 min at 25° C, 2 min at 72° C). Then 50 pmol of primers H3T7 and the reverse primer B (FIG. 5) and an additional 1.3 units of Taq polymerase were added in 10 ul 1X reaction buffer and the amplification continued for 25 cycles (each consisting of 2 min at 94° C, 2 min at 55° C, 2 min at 72° C). The PCR products were extracted with chloroform, precipitated with ethanol, and resuspended in 10 ul RNAse-free water.

EXAMPLE 4.

In vitro transcription of DNA templates

The DNA of interest spliced to the universal promoter was transcribed into mRNA in vitro with the use of a commercially available in vitro transcription kit (Pro-Mega, Madison, WI). This reaction makes mRNA molecules that have at their 5' ends a site for ribosome binding, an enhancer of translational activity and a start AUG codon in correct reading frame with the desired sequence downstream from it. Regions transcribed are not dependent on the presence of restriction enzyme sites, but are dictated by the selection of the original primers in EXAMPLE 2. This allows all molecules to initiate and end at any desired point within the open reading frame of the gene. Extra primers left from the PCR reaction do not have to be removed as they do not interfere with the transcription reaction and do not bind to the native T7 RNA polymerase.

One microliter of DNA template produced by E-PCR (EXAMPLE 3) was added to a 50 ul transcription reaction containing 40mM Tris HCL pH 8.0; 8mM $MgCl_2$; 2mM spermidine; 10 mM NaCl; 10 mM DTT; 40 units of RNasin (Pro-Mega); 500 uM each of ATP, CTP, GTP, and UTP; and 25 units of T7 RNA polymerase (Pro-Mega). The reaction was incubated at 37°C for 60 minutes. The DNA template was digested with 1 unit of RQ1 DNase (Pro-Mega) at 37°C for 15 minutes, followed by phenol extraction, ethanol precipitation, and resuspension in 10 ul RNase-free water.

EXAMPLE 5.

In vitro translation of protein

The mRNA transcribed from the E-PCR DNA template can be translated in vitro using a variety of commercially available systems that include rabbit reticulocyte lysate, wheat germ extract or bacterial extract. These systems each contain the endogenous cellular components necessary for protein synthesis: ribosomes; tRNA; and initiation, elongation, and termination factors. A mixture of amino acids is added, one or more of which can be labeled with a radioactive marker to allow quantitation and analysis of the protein product. The optimum potassium acetate and magnesium acetate levels for each particular mRNA should be determined for highly efficient translation of the mRNA. Additionally, the mRNA may be injected into in vivo translation systems such as frog oocytes. Therefore, following fairly standard protocols radiolabeled protein can be produced which can be used in a functional assay such as precipitation with

specific antibody or binding to a specific receptor.

Specifically, for translation of the mRNA (EXAMPLE 4) in a rabbit reticulocyte system, the mRNA was heated to 67° C for 10 min and immediately cooled on ice. This increases the efficiency of translation, especially of GC-rich mRNA, by destroying local regions of secondary structure. The reagents of the translation mixture were added in a 0.5ml polypropylene microcentrifuge tube (35ul nuclease treated lysate, 7ul water, 1ul RNasin ribonuclease inhibitor (at 40u/ul), 1ul mM amino acid mixture (minus leucine), 1 ul mRNA substrate, 5ul ^{3}H-leucine (100-200Ci/mmole) at 5mCi/ml). This reaction is incubated at 30° C for 60 min.

EXAMPLE 6

Immunoprecipitation of in vitro translated protein with specific antibody

This example illustrates the use of this method to check that a cloned gene is in the correct reading frame or that mutations have not been introduced during cloning manipulations that could alter the reading frame of the expected recombinant product. The protein product of a gene cloned in the correct reading frame produces an epitope that is recognized by antibodies specific to epitopes on that protein.

Using the appropriate primers for the 5' and 3' ends of the gene the DNA segment of interest can be amplified directly, as in EXAMPLE 2, from a bacterial colony and that PCR product can be spliced to the universal promoter-3 as in EXAMPLE 3; transcribed into mRNA as in EXAMPLE 4; and translated into protein as in EXAMPLE 5. This protein can then be immunoprecipitated with antibodies against epitopes specific to the protein (FIG 7).

Specifically, a DNA construct was made of the gene for the circumsporozoite protein of Plasmodium falciparum that did not code for the internal repeated peptides. This construct, called "repeatless", was cloned into a plasmid vector, pADE171 and transformed into the bacteria Salmonella typhimurium. After construction and transformation it was of interest to determine whether or not the gene could encode the correct "repeatless" protein. Production of the protein in Salmonella is minimal under in vitro growth conditions. Therefore, E-PCR was employed to answer the question. A single colony of bacteria on an agar plate was touched with a sterile toothpick and adherent bacteria were lysed by placing the toothpick into 10 ul of 0.1 N NaOH for 10 min at room temperature. The solution was neutralized with 10 ul of 0.5M

Tris, pH 7.5 and the 20 ul added to 980 ul of water. After mixing well, 10 ul of this was used in a standard PCR reaction as in EXAMPLE 2 with a "splicing primer" (T7CS) specific for the 5' portion of the gene and the 3' end of the universal promoter-3 and a reverse primer (RAS 2) specific to the 3' end of the gene of interest. The first 9 nucleotides of T7CS were the same as the last 9 nucleotides of the universal promoter-3 to allow splicing of the product to the universal promoter-3. After the PCR reaction the product was electophoresised in a 0.8% agarose gel and the band containing the amplified DNA cut out and stored in a 1.5ml polypropylene microcentrifuge tube at -20° C. The DNA was spliced to the universal promoter-3 as in EXAMPLE 3; transcribed into mRNA as in EXAMPLE 4; and translated into protein as in EXAMPLE 5 in the presence of ^{35}S-methionine. About 100,000 cpm were added to antibody that was specific to either the repeat region (which should not be made in this construct), the non repeat region, the CS gene product of Plasmodium berghei, or normal rabbit antibody. The mixture was allowed to react for 1 hr at room temperature and then Protein A-Sepharose beads were added for 1 hr. After this time the beads were rinsed and the antibody-protein complex eluted off in 50 ul of 2x SDS gel loading Buffer and 10 ul of this mixture analyzed on a 4 to 20% acrylamide denaturing gel. As shown in Fig. 5 only the antibodies specific to the nonrepeat region of the Plasmodium falciparum CS gene precipitated the gene product. Consequently by E-PCR it was indicated that the gene cloned into Salmonella was in the reading frame to be correctly translated in to the desired protein product.

EXAMPLE 7

Binding studies of in vitro translated E-PCR products

This example illustrates the use of this method to rapidly produce a protein which has a specific functional property, e.g. the ability to bind to red blood cells.

Using the technique with on a plasmid containing the entire EBA-175 sequence a 519 bp gene product was amplified as indicated in EXAMPLE 2 using oligo primers EBA-D (5'-ATGGCATTACGTACGGATGAACGAAA-3') and EBA-E (5'-ACGTGGATCCCTACTCTGTATCAGAACTTC-3'). This DNA fragment encodes a punitive red blood binding domain polypeptide. The DNA fragment was linked to UP3 as indicated in example 3 and the product in vitro transcribed and translated as in EXAMPLES 4 and 5. As shown in FIG 8, lane A, several size products are translated from the mRNA, most probably due to incomplete synthesis by the ribosomes or truncated mRNA molecules. However, as shown in lane B, only full length polypeptide bound to red blood cells.

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The patents and scientific publications referred to herein are considered relevant to the instant invention and said patents are hereby expressly incorporated by reference.

It is understood that the invention is not limited to the particular embodiments specifically disclosed herein as exemplary, but embraces such modified forms thereof as come within the scope of the following claims.

What is claimed is:

1. A method for producing in vitro proteins of a selected DNA sequence without cloning, comprising the steps of:

a. Synthesizing a universal promoter selected from the group consisting essentially of the promoters represented by Figures 1, 2, 3, and 4, each promoter comprised of double stranded DNA having a RNA transcription promoter sequence followed by an untranslated leader sequence that enhances translation activity, followed by three codons the first of which is comprised of the nucleotides coding for the codon of the amino acid methionine;

b. Synthesizing of an oligonucleotide primer whose 3′ sequence is complementary to the 5′ ending of the sense strand of the universal promoter and adds 4 or more nucleotides upstream of the -17 position of the universal promoter;

c. synthesizing an oligonucleotide primer (splicing primer) whose sequence is the same as those nucleotides of the universal promoter beginning with the ATG codon for methionine and having the remaining nucleotides the same as, and in reading frame as, the gene segment of interest said oligonucleotide primer being about 12 to about 21 nucleotides in length to specifically anneal to the gene segment of interest.

d. synthesizing an oligonucleotide primer "reverse primer" whose sequ nce is the same as a segment of the complementary strand of the gene of interest but downstream from the splicing primer such that splicing and reverse primers flank the gene segment of interest;

e. amplifying the gene segment of interest by the polymerase chain reaction using the splicing and reverse primers;

f. splicing and amplifying the universal promoter and the gene segment of interest using a universal promoter of step 1(a) the gene segment of interest of step 1e, primers H3T7 of step 1b and the reverse primer of step 1d by the process known as "splicing by overlap extension-PCR";

g. purifying and concentrating the amplified universal promoter-DNA segment formed in step 1(f) by chloroform extaction and ethanol precipitation and resuspending said universal promoter-DNA segment in an aqueous solution;

h. adding to the universal promoter-DNA of step (g), the phage encoded RNA polymerase specific to the phage promoter in said universal promoter in a RNA generating medium to provide RNA copies of the selected DNA sequence;

i. adding the RNA produced in step (h) to a cell-free or intracellular translation system to provide translation of the RNA into protein.

2. A method according to Claim 1 wherein the double stranded DNA sequence synthesized is th sequenc of the universal promoter-1 represented by Figure 1.

3. A method according to Claim 1 wherein the double stranded DNA sequence synthesized is the sequence of the universal promoter-2 represented by Figure 2.

4. A method according to Claim 1 wherein the double stranded DNA sequence synthesized is the sequence of the universal promoter-3 represented by Figure 3.

5. A method according to Claim 1 wherein the double stranded DNA sequence synthesized is the sequence of the universal promoter-4 represented by Figure 4.

6. A method accoridng to Claim 1 wherein the promoter sequence is each universal promoter of step (a) is the T7 late phage promoter sequence.

7. A method according to Claim 1 wherein the promoter sequence is each universal promoter of step (a) is the promoter sequence recognized by any RNA polymerase.

8. The method of Claim 1 wherein the untranslated leader sequence (UTL) of step (a) is of the alfalfa mosaic virus (AMV).

9. A method according to Claim 1 wherein the untranslated leader sequence (UTL) of step (a) is an untranslated leader sequence of any protein which is shown to enhance translational activity of the downstream sequence.

10. A meth d according to Claim 2 wherein the universal promoter-1 represented by Figure 1 contains three nucl otides upstream of the -17 nucleotide of the T7 promoter and ends with the sequence ATGGCAT.

11. A method according to Claim 3 wherein the universal promoter-2 represented by Figure 2 contains 9 nucleotide upstream of the -17 nucleotide of the T7 promoter.

12. A method according to Claim 3 wherein the universal promoter-2 represented by Figure 2 contains at lease four (4) nucleotides.

13. A method according to Claim 4 wherein the universal promoter-3 represented by Figure 3 contains three nucleotides upstream of the -17 nucleotide of the T7 promoter and ends with the sequence ATGGCACTG.

14. A method according to Claim 5 wherein the universal promoter-4 represented by Figure 4 contains 9 nucleotide upstream of the -17 nucleotide of the T7 promoter.

15. A method according to Claim 5 wherein the universal promoter-4 represented by Figure 4 contains at least four (4) nucleotides.

16. A method according to Calim 1 wherein the oligonucleotide of step (b) is H3T7 represented in Figure 5.

17. A method for producing a universal promoter by the process of primer-dimer formation in the polymerase chain reaction using the two oligonucleotide primers pUP1 and pUP2 represented in Figure 5, comprising the steps of:

a. mixing 100 pm ls each of pUP1 and pUP2 in a final vol of 100 ul of a buffer containing 10 mM Tris-HCl, pH8.3, 50 mM KCI, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 uM each dNTP (dATP, dGTP, dCTP, dTTP) and 2.5 units of Taq DNA polymerase;

b. amplifying the mixture in 20 separate cycles of a polymerase chain reaction, each cycle being 2 min at 94°C, 2 min at 50°C.

c. incubating the product of step b. at 72°C for 7 minutes;

d. extracting the product of step c. one time with 100 ul of cloroform, and adding 50 ul of 7.5M ammonium acetate and 2 vols of 100% ethanol to cause precipitation and placing the resulting at -20°C for 30 minutes;

e. the mixture of step d. was centrifuged, the precipitate washed with cold 80% ethanol and resuspended in 20 ul of TE (10mM tris, pH 8.0, 1mM EDTA);

f. loading the DNA in solution onto an agarose gel consisting of 2% NuSieve agarose, 1% Seakem agarose, 0.5ug/ml ethidium bromide, 1X TAE buffer (10X Buffer is 0.4M Tris Base, 0.2M sodium acetate, 10mM EDTA, pH7.2).

g. subjecting the gel to electrophoresis for 30min in 1X TAE buffer and the band excised and stored in a 1.5ml polypropylene microcentrifuge tube at -20°C.

18. A method according to Claim 17 wherein the universal promoter is used without further purification by melting the gel in the polypropylene microcentrifuge tube at 65°C for 10 min and adding 1 ul of melted agarose and DNA to the SOE reaction.

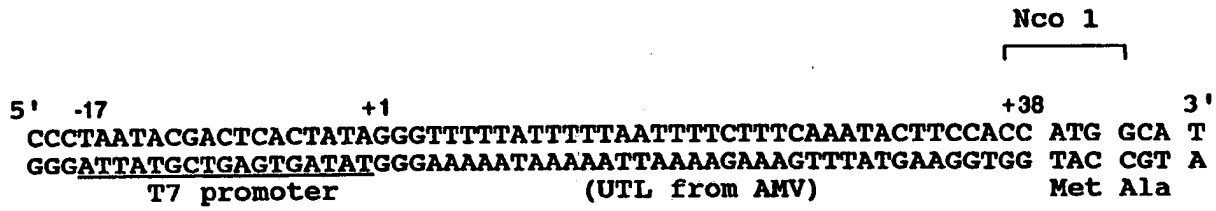
19. A method according to Claim 17 wherein universal promoter is purified and quantified by isolating the fragment from the gel of step g. by electoelution or solid matrix binding (glass milk purification).

20. The universal promoter-1 represented by Figure 1.

21. The universal promoter-2 represented by Figure 2.

22. The universal promoter-3 represented by Figure 3.

23. The universal promoter-4 represented by Figure 4.



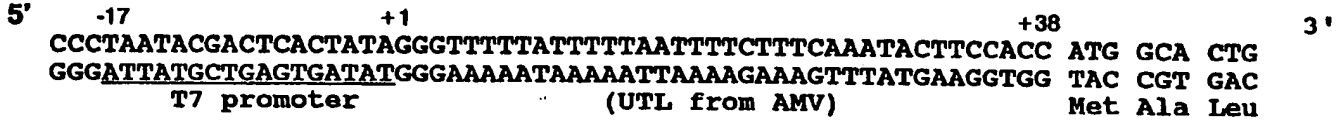
UNIVERSAL PROMOTER - 1

1/2

FIG. 1

UNIVERSAL PROMOTER - 2

FIG 2



UNIVERSAL PROMOTER - 3

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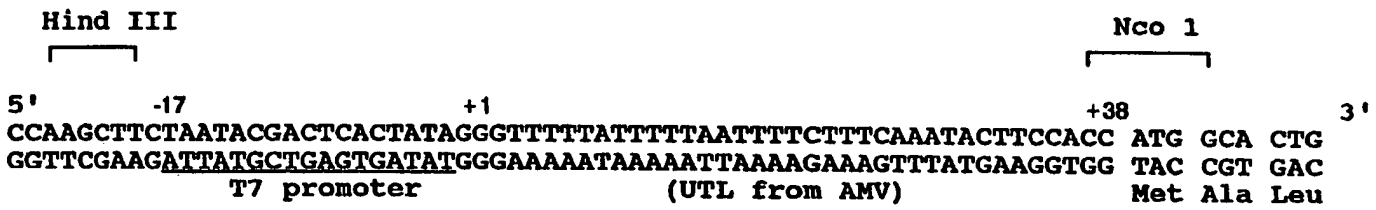
FIG. 3



UNIVERSAL PROMOTER - 4

FIG. 4

SEQUENCE OF PRIMERS USED IN E-PCR

```
     5'                                                          3'
pUP1 CCCTAATACGACTCACTATAGGGTTTTTATTTTTAATTTTCTTTC

     5'                              3'
pUP2 ATGCCATGGTGGAAGTATTTGAAAG

     5'                                3'
pUP3 CAGTGCCATGGTGGAAGTATTTGAAAG

       5'                                        3'
(H3T7) CCAAGCTTCTAATACGACTCACTATAGGG
       HIND III                  T7 promoter

    5'                                  3'
(A) ATGGCATTAGAAGACGTGAAAGAACAT
    UP-1 specific        GENE-specific

    5'                                  3'
(A1) ATGGCACTG GAAGACGTGAAAGAACAT
     UP-3 specific       GENE-specific

    5'                                                  3'
(B) CGATGGATCCCTTGAAAAAGCCTCCTTTCTGAAAC
```

FIG. 5

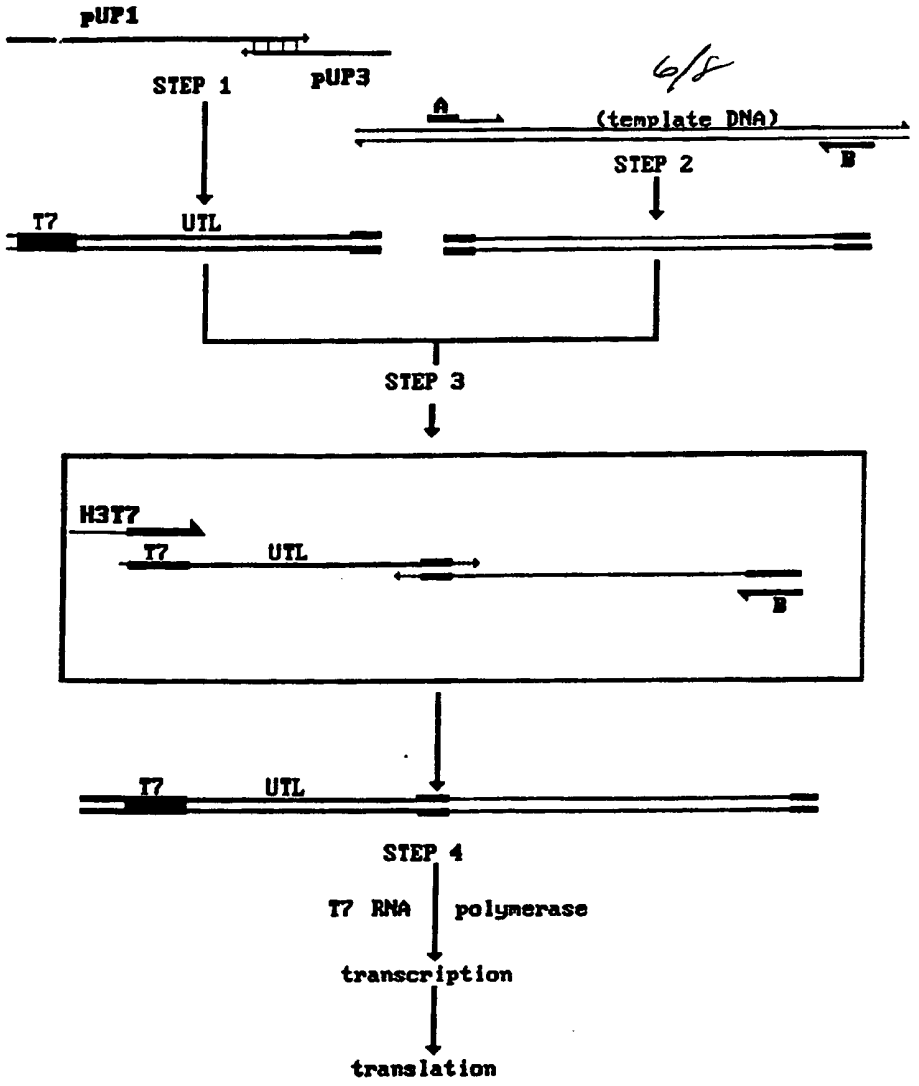

pUP1
pUP3
STEP 1
6/8
A
(template DNA)
B
STEP 2
T7
UTL
STEP 3
H3T7
T7
UTL
B
T7
UTL
STEP 4
T7 RNA polymerase
transcription
translation

Fig 6.

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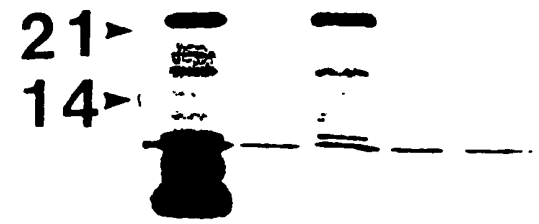

Fig 7

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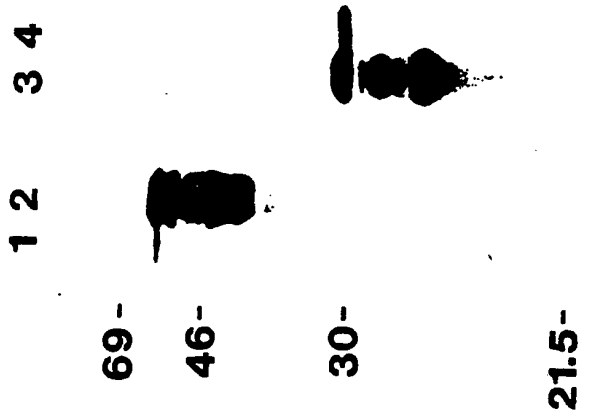

Fig 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08291

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12P 21/02, 19/34; C07H 21/04
US CL : 435/69.1, 91; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/69.1, 91; 536/27

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

CHEMICAL ABSTRACTS

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y,P	US, A, 5,023,171 (Ho et al.) 11 June 1991, see entire document.	1-23
Y	Gene, Volume 77, No. 1, issued 1989, R. M. Horton et al., "Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension", pages 61-68, see entire document.	1-23
Y	Nature, Volume 325, issued 12 February 1987, S. A. Jobling et al., "Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence", pages 622-625, see entire document.	1-23
Y	Science, Volume 239, issued 29 January 1988, E. S. Stoflet et al., "Genomic Amplification with Transcript Sequencing", pages 491-494, see entire document	1-23
A	BioTechniques, Volume 8, No. 2, issued February 1990, D. H. Jones et al., "A Rapid Method for Site-Specific Mutagenesis and Directional Subcloning by Using the Polymerase Chain Reaction to Generate Recombinant Circles", pages 178-180, 182-183, see entire document.	1-23

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
11 MARCH 1992	20 MAR 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	JAMES KETTER